TELOPHASE ENUCLEATED OOCYTES FOR NUCLEAR TRANSFER

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The present invention relates to an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals, and to a method of reconstituting an animal embryo.

(b) Description of Prior Art

The technique of nuclear transfer has been widely used to multiply embryos by transferring blastomere nuclei from early-stage embryos into enucleated oocytes. This technique enables an increase in the yield of embryos produced from parents of top genetic value, enabling to accelerate the annual genetic gain within an animal population. Nuclear transfer has also been used with nuclei from cell lines derived from embryonic (Campbell et al., 1996, *Nature* **380**:64-66), fetal and adult tissue (Wilmut et al., 1997, *Nature* **385**:810-813). By using nuclei from an unlimited source, nuclear transfer from cell lines enables not only the production of a larger number of genetically identical offspring but also an opportunity for modifying the genetic characteristic of cells *in vitro* prior to the production of live offspring, enabling the production of transgenic mammals. Moreover, the use of cells from adult animals for nuclear transfer, either directly or through previous *in vitro* passage, enable the multiplication (cloning) of animals of known phenotypes.

Basically, the nuclear transfer technique requires a donor nucleus to provide the genetic material of choice and a host oocyte to provide the cytoplasm that plays a role in reprogramming the

nucleus to support embryo development. With the nuclear and cytoplasm sources in hand, three main steps are required to reconstruct an oocyte by nuclear transfer. First, host oocytes need to be enucleated to remove all nuclear genetic material. This step is usually performed by microsurgical removal of the chromosomes from either a metaphase plate or pronuclei. Second, donor nuclei need to be introduced into the oocyte (nuclear transfer). This step is normally obtained by fusing the membranes of the nuclear donor cell and the host oocyte. However, nuclear transfer can also be obtained by traversing the oocytes plasma membrane and microinjecting the nucleus directly into the host cytoplasm. Finally, non-activated host oocytes need awakening from their meiotic arrest (oocyte activation). This step can be achieved by exposing the oocyte to a physical stimulus, such as temperature changes or an electric shock, or exposing the oocyte to chemical agents, such as ethanol or exogenous calcium. The order in performing each of the steps above can vary in different situations and may have an important effect on the ability of the reconstructed oocyte to undergo further development.

In mice, oocyte enucleation was performed after fertilization by visualizing and removing the pronuclei by microsurgery. This enucleation technique is less efficient in other mammals due to the higher density of cytoplasm resulting in poor visualization of pronuclei. Moreover, attempts to use pronuclear-stage enucleated oocytes led invariably to poor developmental rates when using cleavage stage blastomeres as nuclear donors. Improved development after nuclear transfer was achieved initially in sheep (Willadsen,S. 1986, *Nature* **320**:63-65) and later in other mammals using host oocytes that had not been activated at the time of

fusion. However, a problem remained that metaphase stage chromatin cannot be visualized easily by microscopy in most mammals. Willadsen (Willadsen, S.1986, *Nature* **320**:63-65) proposed an enucleation procedure in which sheep oocytes were blindly divided into halves either containing or not the first polarbody. To avoid a large loss of cytoplasm during enucleation, this procedure was later improved by using a DNA vital stain (Bisbenzimide; Hoechst) and ultraviolet (UV) irradiation to check whether the MII plate after removal of small portions of cytoplasm. The most common procedure of oocyte enucleation is to expose secondary oocytes to bisbenzimide, blindly remove a cytoplasmic fragment surrounding the first polarbody and then expose the oocyte to UV to ascertain whether enucleation was correctly performed. On average this procedure correctly enucleates between 60 to 80 percent of oocytes. Another possible limitation of this procedure is that oocytes are exposed both to UV irradiation and Hoechst 33342 that have been shown to have detrimental effects on the cytoplasm (Smith,L. 1993 *J. Reprod. Fert.* **99**:39-44).

As mentioned above, host oocytes are able to support better development after nuclear transfer when compared to pronuclear-enucleated host zygotesIt has already been shown that MII-stage enucleated oocytes either aged or activated before fusion support better development. The problem of using young non-activated oocytes is caused by incompatibilities between the cell cycle stages of the nuclear donor cell and the host cytoplasm. Metaphase arrested secondary (MII) oocytes have high levels of a Maturation Promoting Factor (MPF), a cellular activity that is responsible for maintaining the chromatin condensed without a nuclear envelop. When blastomere interphase-stage nuclei

containing decondensed chromatin are introduced into an MII oocyte, MPF leads to a rapid breakdown of the nuclear membrane and premature chromosome condensation (PCC). However, PCC is believed to be detrimental only when induced during the DNA synthesis stage (S-phase) of cell cycle. This is particularly problematic when using donor nuclei from blastomeres since these undergo S-phase for most time in between cell divisions. On the other hand, enucleated oocytes that have been activated or aged before fusion to nuclear donor cells have lower levels of MPF and, therefore, do not cause PCC.

With the exception of blastomeres, most other cell types have longer gaps both before (G1-phase) and after (G2-phase) the S-phase and, therefore, are less susceptible to the harmful effects of S-phase PCC when fused to a MII oocytes. Because high MPF levels cause the breakdown of the nuclear membrane, MII stage host oocytes are believed to facilitate interactions between donor nuclei and putative oocyte cytoplasmic 'factors' required for reprogramming the chromatin of nuclei derived from cells further advanced in differentiation. Several examples in the literature report on the advantages of passaging further differentiated donor nuclei in non-activated MII oocytes before activating the reconstructed oocyte. In cattle, nuclei from an embryonic cell line supported significantly higher yield of blastocyst development and more 30d pregnancies when fused to enucleated oocytes 4 h before activation. In mice, significantly more embryos reconstructed with cumulus cell nuclei developed to the blastocyst stage by exposing the donor nucleus to MII cytoplasm for between 1 and 6 h before activation (Wells et al. 1999, *Biol. Reprod.* **60**:996-1005). Moreover, no fetal development or live offspring was obtained when using with simultaneous activation and

fusion. Furthermore, other reports using differentiated cell lines have used host oocytes that were either activated after or concurrently with introducing the donor nucleus (Cibelli et al. 1998, *Nature Biotechnol.* **16**:642-646; Wilmut et al. 1997, *Nature* **385**:810-813). Therefore, the prevalent theory in the field of cloning by nuclear transfer is that a period of reprogramming in the cytoplasm of an inactivated oocyte is required to obtain success when using donor nuclei from cells other than embryonic blastomeres.

It would be highly desirable to be provided with an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

It would be highly desirable to be provided with an improved method of reconstituting an animal embryo.

SUMMARY OF THE INVENTION

The present invention described below is contrary to current knowledge in that we are teaching use of an activated oocyte as recipient for nuclei derived from cells from embryonic and somatic cell lines.

One aim of the present invention is to provide an improved method for obtaining an enucleated host oocyte for transferring nuclei from embryonic, germinal and somatic cells with the objective of cloning or multiplying mammals.

Another aim of the present invention is to provide an improved method of reconstituting a non-human embryo.

In accordance with the present invention there is provided a method of preparing an enucleated host

oocyte for transferring nuclei from embryonic, germinal or somatic cells, which comprises the steps of:

a) activating oocyte by artificial means; and

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II); and

c) transferring nuclei from embryonic, germinal or somatic cells into the enucleated oocyte of step b), wherein embryonic cells are cultured prior to nuclei transfer.

The germinal or somatic cells are cultured prior to nuclei transfer.

The oocyte of step a) has a first polarbody and the artificial means is chemical means, such as ethanol or ionomycin.

Step b) may be performed after oocytes are cultured for a period of time sufficient to allow for extrusion of a second polarbody.

Step b) may be performed with oocytes in a medium with cytosqueletal inhibitors.

Step b) may be effected by microsurgically removing the second polar with about one tenth of the cytoplasm surrounding the second polarbody.

The preferred oocyte is a secondary (M-II) oocyte.

In accordance with the present invention, there is provided a method of reconstituting a non-human embryo, which comprises the steps of:

a) activating oocyte by artificial means;

b) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte

has recently expelled second polarbody (Tel-II);

c) transferring a diploid nucleus in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

d) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

In accordance with the present invention, there is provided a method for production of a transgenic non-human embryo, which comprises the steps of:

a) transfecting cultured cells selected from the group consisting of embryonic, germinal and somatic cells with a desired DNA construct;

b) activating oocyte by artificial means;

c) enucleating the activated oocyte when the activated oocyte is undergoing the expulsion of a second polarbody or when the activated oocyte has recently expelled second polarbody (Tel-II);

d) transferring a diploid nucleus extracted from the transfected cells of step a) in the enucleated oocyte to obtain a reconstructed oocyte with a diploid chromosomal content; and

e) culturing *in vitro* the reconstructed oocyte and/or transferring the reconstructed oocyte into a reproductive tract of a suitable surrogate mother to enable development into a non-human embryo.

The non-human embryo may develop into a non-human animal.

BRIEF DESCRIPTION OF THE DRAWING

Fig. 1 illustrates a schematic protocol of the technique of telophase enucleation for nuclear transfer.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of producing embryos by nuclear transplantation from embryonic, germinal and somatic cells lines. Nuclear transfer procedures have invariably initiated with the enucleation of host oocyte. The enucleation procedure is followed by one of the following: (a) activation followed by fusion; (b) concurrent activation and fusion; or (c) fusion followed by activation. Whereas the procedure in which oocytes are (a) enucleated, activated and then fused is used mostly for embryonic blastomeres, most techniques applied for further differentiated donor nuclei use the procedure where oocytes are enucleated, (b) fused and activated concurrently or (c) fused and later activated. Although the different approaches in the nuclear transfer procedure have been described previously (U.S. Patent No. 4,994,384; U.S. Patent No. 5,057,420; U.S. Patent No. 5,843,754 and International Patent applications Nos. PCT/GB96/02098, PCT/US98/00002, PCT/US98/12800, PCT/US98/12806, and PCT/US97/12919), the present invention describes a sequence of steps in the nuclear transfer procedure that is novel (Fig. 1).

As illustrated in Fig. 1, Step 1 involves the activation of secondary (M-II) oocytes by artificial means. Step 2 is performed shortly after activation when the oocyte is undergoing the expulsion or recently expelled the second polarbody (Tel-II). Step 3 relates to the transfer of a nucleus from any source with the

purpose of reconstructing the oocyte with a diploid chromosomal content.

Step 1 (oocyte activation)

Oocytes are obtained either *in vivo* or *in vitro* and cultured in maturation medium. After maturation, oocytes are denuded of cumulus cells and those with a first polarbody are parthenogenetically activated by chemical means using ethanol or ionomycin. After activation, oocytes are cultured for a few hours to allow for extrusion of the second polarbody.

Step 2 (oocyte enucleation)

After activation, oocytes can be placed in medium with cytosqueletal inhibitors to facilitate microsurgery. Only oocytes with a second polarbody extruded or partially extruded are used. Approximately one tenth of the cytoplasm surrounding the second polar body is microsurgically removed with the second polarbody.

Step 3 (nuclear transfer)

After enucleation, a single cell containing a diploid nucleus is introduced into the enucleated oocyte either by cell fusion or microinjection (nuclear transfer). The reconstructed oocyte is then cultured *in vitro* and/or transferred into the reproductive tract of a suitable surrogate mother to enable further development.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE 1

Telophase Enucleation

Follicles with 2 to 8 mm diameter were aspirated from bovine slaughterhouse ovaries. Oocytes with a homogeneous cytoplasm and several layers of cumulus cells were selected and placed in maturation within 1 h from follicular aspiration. At 28 h after maturation oocytes were denuded of cumulus cells and those with a first polarbody were used in the experiment. Oocytes were exposed to 7% ethanol for 5 min, washed and placed in maturation medium for different periods. At 1 h before microsurgery, oocytes were placed in cytochalasin B and positioned for micromanipulation. Oocytes undergoing extrusion or already with extruded second polarbodies had 10% of their cytoplasmic volume removed together with the second polarbody. After microsurgery, oocytes were fixed in 10% formalin, stained with 5 µg Hoechst 33342 and observed under UV epi-fluorescence. Oocytes without any chromatin were considered successfully enucleated. Most oocytes were successfully enucleated when micromanipulated at the times examined (Table 1). Because the efficiency of this enucleation technique is high, checking of oocytes with DNA stain and UV light is not necessary. Significantly lower percentages of enucleation was obtained when blindly removing using the position of the first polarbody to aspirate 30% of the surrounding cytoplasm in oocytes at metaphase (59%) at 24 h from the beginning of *in vitro* maturation.

Table 1

Successful telophase enucleations as performed at different times after exposure to a stimulus to parthenogenetically activate secondary oocytes

	Time after activation			
	3 h	4 h	5 h	Total
Number manipulated	37	38	43	118
Successful enucleation (%)	36 (97%)	37 (97%)	40 (93%)	113 (96%)

Example 2

Nuclear transfer with morula-stage blatomeres

Bovine secondary oocytes were matured *in vitro* and enucleated using the technique described above (telophase enucleation). Morula-stage embryos were disaggregated and individual blastomeres were inserted into the perivitelline space of enucleated oocytes. Fusion between the membranes of blastomeres and oocytes was obtained with an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 60 µsec pulses of 1.5 KVolts per cm. After fusion the embryos were cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 2

Fusion and development of bovine oocytes reconstructed with nuclei from morula-stage blastomeres recovered 5 days after IVF

	Number	Fused	Blastocyst	No. nuclei
Telophase II (%)	215	129 (58%)	49 (38%)	126±11
Metaphase II (%)	248	151 (60%)	24 (16%)	84±9

Example 3

Nuclear transfer with non-starved bovine ES cells

Bovine embryo stem (ES)-like cells were obtained from day 8 blastocyst stage embryos produced entirely *in vitro*. ICMs were platted onto mitomycin-inactivated mouse fibroblasts. Established ES-like lines were disaggregated by short exposure to trypsin using a narrow pipette. Isolated cells were placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse that causes fusion between the membranes of the donor and recipient cells. The electrical parameters used were double 100 µsec pulses of 1.5 KVolts per cm. Electrical stimulation was performed as soon as possible after placing the nuclear donor cell in the perivitelline space to obtain better fusion results. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 3

Fusion and development of bovine oocytes reconstructed with nuclei from ES-like cells exposed to 5% of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II (%)	38	11 (30%)	5 (45%)	3 (27%)
Metaphase II (%)	33	12 (36%)	2 (17%)	1 (8%)

Example 4

Nuclear transfer with serum-starved bovine ES cells

Bovine embryo stem (ES)-like cells were cultured in medium with 0.5% FCS for 5 days before micromanipulation. As described above, ES-like cells were disaggregated, placed in the perivitelline space of enucleated oocytes and exposed to an electric pulse to cause fusion between the membranes of the donor and

recipient cells. After fusion the embryos are cultured for 7 days in the presence of Menezo's B2 medium supplemented with 10% fetal calf serum.

Table 4

Fusion and development of bovine reconstructed with nuclei from bovine ES-like cells exposed (starved) to low concentrations (0.5%) of FCS

	Number	Fused	Cleaved	Blastocyst
Telophase II(%)	38	13(34%)	3(23%)	2(27%)
Metaphase II(%)	42	13(31%)	4(31%)	1(15%)

Example 5

Nuclear transfer with starved and non-starved bovine fetal fibroblasts

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. Non-starved fibroblast cells were recovered during growth at 2 days after passaging. Serum starved cells were exposed to medium with 0.5% serum for 5 days before NT. NT was performed as described above.

Table 5

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells exposed for 5 days to low concentrations (0.5%) of FCS (starved) or to 5% FCS for 20 h after seeding (non-starved)

	Serum starved			Non-starved		
	Number	Fused	Blast.	Number	Fused	Blast.
Telophase II (%)	69	52 (75%)	2 (4%)	105	67 (64%)	9 (13%)
Metaphase II (%)	60	39 (65%)	9 (24%)	114	92 (81%)	12 (13%)

Example 6

Nuclear transfer with starved and non-starved bovine fetal fibroblasts transfected with a GFP construct

Bovine fetal fibroblast cells were recovered form day 50 fetuses and passaged in medium D-MEM with 10% FCS. The fetal fibroblast cells were transfected with a constructs containing the CMV/eGFP gene (plasmid pGREEN LANTERN-1, Life Technologies). This construct contains the reporter gene Green Fluorescence Protein (GFP) from Aequorea victoria jellyfish, which codes for a naturally fluorescent protein requiring no substrate for visualization. The GFP used is "humanized" (ie., codon sequence) and mutated to contain threonine at position 65 to enhance fluorescence peaking. The advantage of using this fluorescent gene as a reporter being that it yields bright green fluorescence when living or fixed cells are illuminated with blue light and increases our sensitivity of detection. The plasmid contains the CMV immediate early enhancer/promoter upstream of the GFP gene, followed by SV40 t-intron and polyadenylation signal. NT was performed as described above.

Table 6

Fusion and development of bovine reconstructed with nuclei from bovine fetal fibroblast cells transfected with a GFP construct and starved for 4 days and transferred to metaphase stage-enucleated oocytes or cultured for 6 h after thawing and transferred to telophase stage-enucleated oocytes

	Number	Fused	Blastocyst
Telophase II(%)	187	131(71%)	15(11%)
Metaphase II(%)	209	169(81%)	23(14%)

Table 7

Post-implantation development of cloned blastocyts derived from GFP-positive fetal fibroblasts (Table 6)

	No Embryos	No Recipients	Non-returned	60 d positive	200 d positive	liveborn
Telophase II(%)	11	6	2	1	1	1
Metaphase II(%)	15		5	4	4	3

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.